Report of Dr. Avery (assisted by Drs. Hotchkiss, McCarty and Taylor

Studies on the transformation of pneumococcus (Avery, McCarty and Taylor). The significance of the phenomenon of transformation lies in the fact that heritable and specific alterations in structure and function of unencapsulated (R) variants can be selectively brought about by highly purified desoxyribonucleic acid fractions isolated from encapsulated strains of different specific types. The changes induced are not random but are predictable and predetermined according to the specific type of Pneumococcus used as source of the transforming substance. Transformation involves the synthesis of a new capsular polysaccharide chemically and serologically different from that known to be produced by the encapsulated cells from which the variant strain was derived. Once transformation has occurred the newly acquired characters are thereafter transmitted to daughter cells through innumerable transfers in culture media without further addition of the transforming substance. From the transformed cells, a nucleic acid fraction having identical chemical properties and biological activity can again be recovered in amounts greatly in excess of that used to initiate the reaction.

It is evident that not only is the capsular polysaccharide reproduced but the inducing substance which determines the synthesis and specificity of the capsule is also reduplicated in successive generations.

Various interpretations have been advanced as to the nature of this phenomenon. However, those of us actively engaged in the work have for the most part left matters of interpretation to others and have chosen rather to devote our time and thought to experimental analysis of the factors involved in the reaction. This is not to say that we are

indifferent and have not among ourselves indulged in speculation and discussion of the relation of the problem to other similar phenomena in related fields of biology. Moreover, considerable doubt has been expressed as to the chemical identity of the transforming substance. From the beginning we ourselves have been keenly alert to the possibility that the presence of some substance other than the desoxyribonucleate in our preparations may be responsible for biological activity. Thus far, the accumulated evidence strongly supports the belief originally expressed that "a nucleic acid of the desoxyribose type is the fundamental unit of the transforming substance". Nevertheless, this question is of such importance that it is hoped that it may be made possible to continue the work on this and other chemical aspects of the problem.

Biochemical studies of environmental factors essential in transformation (Hotchkiss and Taylor). One of the major problems under investigation at present concerns the chemical nature and biological activity of certain components in serum which are essential in the transforming reaction. In the preceding report, Dr. McCarty described a method in which the specific enzyme, desoxyribonuclease, is used as a biochemical tool for determining the time required for the transforming principle to be taken up or "fixed" by competent R cells during growth in the presence of serum. Using this method it can be readily shown that the addition of the enzyme to the reaction system at any time up to 3-4 hours after inoculation interfers with the reaction so that transformation does not occur. It appears that throughout this initial period the transforming substance has not yet been taken up but is still free in the medium and hence subject to the destructive action of the enzyme. On the other hand, if the enzyme is added later than 4-5 hours after inoculation, it has no effect on the

subsequent development of transformation. The results indicate that a critical growth period of from 3-5 hours in serum medium is required before the specific desoxyribonucleate is taken up by the R cells and consequently protected from destruction by the enzyme.

These observations have been confirmed by the results of corollary experiments in which R cells are first grown 4-5 hours in serum broth alone and then transferred to medium containing the transforming substance. Under these conditions it has been found that the serum treated cells are now capable of taking up the transforming substance within 5 minutes after inoculation in contrast to the 4-5 hours normally required when untreated cells are used as inoculum. It therefore appears that in transformation the primary action of the serum is on the R cells as a result of which they become capable of interacting with the transforming principle. Although the mechanism of this action is as yet unknown, its effect is manifest by the readiness with which "sensitized" cells take up the specific desoxyribonucleate. For purposes of convenience and without any implication as to its nature, this preparatory process is referred to as "sensitization" and the potency of whole serum or a serum fraction can be defined in terms of its "sensitizing activity." It is thus apparent that the interaction between competent R cells and the specific transforming substance is mediated by serum, the function of which is to prepare R cells for the rapid uptake of the transforming principle. As will be discussed below, it has been found recently that a crystalline protein isolated from human and bovine sera possess sensitizing activity comparable to that of the action of whole serum.

In the last report it was tentatively proposed that human serous fluids provided at least three important components: a) Agglutinins for R

pneumococci which serve the purpose of causing essential colonial aggregation. These aggregates of agglutinated cells in turn are thought to create in their environment the local conditions, possibly reducing in character, that are required for transformation; b) A non dialyzable factor, presumably protein in character, which had not at that time been sufficiently defined to establish its chemical identity. The fact that serum can be completely inactivated by prolonged dialysis suggested the existence of (c) a dialyzable component. A primary concern of this laboratory during the past year has been to clarify the nature of the last two components.

As previously reported, a serum inactivated by prolonged dialysis regains its activity on incubation with inorganic phosphate. It has since been found that reactivation of dialyzed serum can be immediately brought about without incubation by the addition of sodium pyrophosphate. A few organic phosphates have been tested but thus far none has been found to have this effect.

As progress has been made in the fractionation of serum, pyrophosphate has proved to be a useful reagent for eliciting the maximal sensitizing activity of serum fractions. A further useful step has been the development of a semi-quantitative method of assay for the serum factor. Briefly, the procedure is as follows: Graded amounts of serum or protein fractions are tested in an enriched broth containing optimal quantities of pyrophosphate, purified R antibodies, and transforming substance. Under these standard conditions, the amount of serum component added becomes the limiting factor, and the rate and extent of occurrence of pneumococcal transformation serve as a satisfactory measure of the sensitizing activity of this component.

The protein fractions tested by this method were prepared either

by the usual salting out procedures or by the newer alcohol fractionation method devised by Professor Cohn of Harvard Medical School. The evidence clearly indicates that the albumin fraction of normal serum of human and bovine origin, and of pleural fluids are potent sources of the non dialyzable protein factor. As little as 300 micrograms of purified serum albumin per cubic centimeter can be the decisive factor determining prompt and typical transformation.

That the sensitizing activity just described may reasonably be attributed to one or other of the serum albumins is indicated by the following observations. Four preparations of crystalline albumin isolated from bovine serum by the Cohn procedure, and considered to be electrophoretically homogeneous, have proved to be as active as are the crude albumin fractions. In addition, a sample of human serum albumin which had been recrystallized four times and found to be electrophoretically homogeneous was likewise active in supporting transformation.

A further indication that sensitizing activity is a property of serum albumin is afforded by the following experiments. When a solution of albumin is heated under various conditions of hydrogen ion concentration and temperature, its sensitizing activity diminishes progressively as more and more of the protein becomes altered in solubility. In very acid solutions (pH 2) albumin is remarkably stable. At this acid reaction solutions of albumin may be heated in boiling water for 15 minutes, cooled, and reneutralized without apparent change in solubility or sensitizing activity. On the other hand, a precipitate is formed when a solution of albumin is heated at 62°C. in the range of its isoelectric point. The supernatant fluid has little or no sensitizing activity. When, however, the precitate is dissolved in hydrochloric acid (pH 2) and the solution

is then neutralized the protein regains its original solubility and the sensitizing activity is restored. Thus, there is a striking parallelism between the native solubility of bovine serum albumin and its sensitizing activity in the transforming system.

Before testing human serum or serous fluids for their ability to support transformation, it has been customary to subject them to heating (60°C. for 30 minutes) at their normal slightly alkaline reaction. This was done in order to inactivate the serum enzyme, desoxyribonuclease, which would otherwise depolymerize and thus destroy the transforming activity of the specific nucleic acid present in the transforming system. It is not necessary to heat purified serum albumin for this purpose, but when it is heated under these conditions, the protein becomes more readily precipitable at lower concentrations of salt. Accordingly, it appears not unlikely that the activity previously observed in globulin fractions of the serum may have been due to such altered albumin.

Although albumin is by far the most active of the serum proteins, it is not possible to state with certainty that it bears the total sensitizing activity of whole serum. While in many instances the activity level of whole serum or a serum fraction appears to be commensurate with its albumin content, there is now experimental evidence that sensitization by albumin known to be active can in some way be masked or blocked by components associated with the globulin constituents. This blocking action may appear when an active albumin is mixed with the globulin fractions of normal serum. It is known that normal sera, which naturally contain their full complement of albumin, vary markedly in their sensitizing activity. It is possible that their action is masked as a result of the interactions mentioned above. This "blocking" phenomenon is under investigation at

present and it may furnish an explanation of the variable results previously observed with certain sera and serum fractions.

Indeed, the reactivation of a dialyzed serum by inorganic phosphate or pyrophosphate may, as previously suggested, be regarded as the restoration, directly or indirectly, of a dialyzable cofactor. Alternatively, it may also be looked upon as the removal of an inhibiting substance the presence of which is indicated by the experiments mentioned above. An inhibitor may combine with the albumin during the redistribution of constituents that follows the loss of electrolytes when such a complex system as serum is dialyzed. The observation that albumin more or less free of other serum constituents does not appear to be inactivated by dialysis but is nevertheless in some instances brought to higher sensitising activity by the addition of pyrophosphate, is in harmony with the second view.

In summary, the results of analysis of the role of serum in the transforming reaction indicate that albumin is the principal and effective component of sera, which, in conjunction with R cell agglutinins, supports pneumococcal transformation.

Transformation studies on several spontaneously appearing variants of Pneumococcus (Taylor). Pneumococci exhibit marked variability in colonial forms covering a wide range of differences in contour, morphology and surface topography. The colonial variants display all degrees of smoothness and roughness from the glistening mucoid to the extremely granular and pebbled surfaced colony. These physical features are in many instances reflected in recognized differences in biochemical and physiological properties of the cells. Considerable information is available with regard to alterations in the antigenic structure,

serological behavior and virulence associated with these colonial changes. It seemed possible that the principles and techniques of transformation might afford a specific means of investigating the hereditary basis of this phenomenon.

Indeed, by the application and extension of the methods used in transformation it has been possible to determine with reasonable assurance that the intermediate smooth organisms arising from mucoid forms of Type III represent cells in which the SIII transforming determinant itself has become modified. These intermediate smooth variants are reminiscent of allelic series in higher organisms.

The property of extreme roughness (ER) as opposed to moderate roughness (MR) is apparently due to the lack of a factor or factors which can be experimentally introduced into the cells by the techniques of transformation. The MR factor is present in the desoxyribonucleic acid fraction of the moderately rough variant and of Type III organisms as well. The factor (MR) which determines the change from extreme to moderate roughness can be introduced independently of the SIII factor. Indeed, the ER forms cannot be transformed to encapsulated Type III organisms until they have first been converted into MR cells; that is, the MR factor must be introduced first, (ER--> MR--> SIII). These observations furnish experimental evidence in favor of the view previously expressed that the desoxyribonucleic acid fraction derived from Type III pneumococci although chemically homogeneous contains molecules of biologically diverse specificities.

This form of analysis is being pursued with three objectives in mind. First, it is hoped that some information may be gained on the nature of "competence", that property of cells which determines their

capacity to respond to a given specific stimulus or factor in transformation. Second, such experiments may make possible further analysis of the nature of the specificity of the transforming substance. Third, the demonstration that two factors, one for moderate roughness (MR), the other for synthesis of capsular polysaccharide (SIII), each coexisting in the same preparation of nucleic acid, can be selectively taken up by pneumococcal cells and independently bring about development of the corresponding traits, justifies the hope that the pneumococcal cell may prove a delicate tool for the study of many hereditary characters at a biological level heretofore unrealized by geneticists.

Crystallization of the C-reactive protein (McCarty). In the past, several studies have been reported from the laboratories of the Department of Respiratory Diseases on the occurrence during acute infections of a protein not normally present in the blood. This protein is designated as the C-reactive protein since it is precipitated from acute phase human sera by the addition of the somatic C polysaccharide of Pneumococcus. Although the precipitation reaction between the C-reactive protein and the C polysaccharide is superfifially analagous to an antigen-antibody reaction, it was shown to differ from the latter in several important respects. Briefly stated, these differences are as follows: (1) The occurrence of the protein is non-specific with respect to the inciting agent of the disease; (2) The protein is present early in the acute phase and disappears rapidly with the onset of convalescence; (3) In contrast to antibodies, it occurs in the albumin rather than the globulin fraction of serum; and (4) The presence of calcium ion is required in the precipitation reaction between the protein and C polysaccharide. The precipitation appears to be a phenomenon based on a

chance complementary relationship between the molecular configuration of the two reacting substances. It is felt that the occurrence of the protein in the blood may be in some way related to the host reaction to infection.

The present studies deal with the isolation of the C-reactive protein in crystalline form. Human serous fluids, i.e., chest and abdominal fluids accumulating in the course of acute infectious processes, have been employed as source material. After initial fractionation with ammonium sulfate, the C-protein is separated from the bulk of serum proteins by precipitation with the C polysaccharide. Dissociation of the polysaccharide-protein complex and crystallization of the protein depend on the use of concentrated sodium sulfate solutions. Recrystallization of the material in the form of symmetrical rhomboid plates occurs readily in the presence of sodium sulfate at 0.75 saturation. The concentration of C-protein in serum is relatively low, and less than 100 mg. of recrystallized protein was obtained from 1.5 liters of chest fluid.

Preparations of the crystalline protein contain none of the C-polysaccharide employed in the isolation, and indeed solutions of the material give the characteristic precipitation reaction when mixed with the polysaccharide in the presence of traces of calcium ion. The crystalline protein is insoluble in distilled water, but dissolves readily in physiological salt solution.

Evidence regarding the purity of the crystalline protein has been obtained by the use of immunological techniques. The protein is highly antigenic for rabbits, and antisera react in high titer with the purified antigen as well as with acute phase human sera which contain the C-reactive protein. On the other hand, the antisera give no reaction

whatsoever with normal human sera. This finding indicates that no appreciable amount of normal serum protein was present in the crystalline C-protein used for immunization of the animals. The antiserum provides a useful tool for further studies, since it serves as a sensitive and selective reagent for detecting the presence of C-reactive protein in minute amounts.

Publication

McCarty, M. Chemical nature and biological specificity of the substance inducing transformation of pneumococcal types. Bact. Rev., 1946, 10, 63.